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Enzymatic modification of low density lipoprotein by purified lipoxygenase plus phospholipase A_2 mimics cell-mediated oxidative modification

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Abstract Low density lipoprotein (LDL) can be oxidatively modified by cultured endothelial cells or by cupric ions, resulting in increased macrophage uptake of the lipoprotein. This process could be relevant to the formation of macrophage-derived foam cells in the early atherosclerotic lesion. The mechanism of endothelial cell modification of LDL is unknown. In the present work we show that incubation of LDL with purified soybean lipoxygenase, in the presence of pure phospholipase A_2, can mimic endothelial cell-induced oxidative modification. Typically, incubation with lipoxygenase plus phospholipase A_2 caused: *1)* generation of about 15 nmol of thiobarbituric acid-reactive substances per mg of LDL protein; *2)* a 4- to 7-fold increase in the rate of subsequent macrophage degradation of the LDL; *3)* a 10-fold decrease in recognition by fibroblasts; *4)* a marked increase in electrophoretic mobility in agarose gels; and, *5)* disappearance of intact apoprotein B on SDS polyacrylamide gels. Degradation of the enzymatically modified LDL by macrophages was competitively inhibited by endothelial cell-modified LDL and by polyinosinic acid, but only partially suppressed by acetylated LDL. The lipoxygenase plus phospholipase A_2-induced modification of LDL is not necessarily identical to endothelial cell modification, but it is a useful model for studying the mechanism of oxidative modification of LDL. This work also represents the first example of oxidative modification of LDL by specific enzymes leading to enhanced recognition by macrophages.—**Sparrow, C. P., S. Parthasarathy, and D. Steinberg.** Enzymatic modification of low density lipoprotein by purified lipoxygenase plus phospholipase A_2 mimics cell-mediated oxidative modification. *J. Lipid Res.* **1988. 29: 745–753.**

Supplementary key words macrophage • scavenger receptor • cholesterol • apoprotein B • fibroblasts • apoB/E receptor • lipid peroxides • atherosclerosis

Macrophage-derived foam cells are an important feature of the early atherosclerotic lesion (1, 2). Native LDL, taken up through the apo B/E receptor, is not an effective cholesterol donor for foam cell formation in vitro (3). Oxidatively modified LDL (4), or LDL whose lysine residues have been chemically modified, e.g., by acetylation (5), can cause foam cell formation via uptake through the macrophage acetyl-LDL (scavenger) receptor. Oxidation of LDL can be effected by a variety of cell types (6–10), or by incubation of LDL with cupric ions (11) or thiol compounds (12). When the oxidative modification leads to increased macrophage degradation of the lipoprotein, it is termed "biological modification." Oxidative LDL modification is a complex process, and is accompanied by the production of thiobarbituric acid-reactive substances (TBARS) (13), increased agarose gel electrophoretic mobility (9), increased buoyant density of the lipoprotein particle (9), hydrolysis of phosphatidylcholine (PtdCho) to 1-monoacyl-*sn*-glycero-3-phosphocholine (13), and breakdown of the apoB protein moiety (13, 14).

The detailed mechanisms of cell-mediated oxidation of LDL are unknown. There has been substantial interest in the potential role of activated oxygen species generated by the cells and released into the medium (8, 10, 15, 16), but no experiment to date has proven that the presence of these compounds is sufficient for LDL modification. An alternative hypothesis is that some oxidative enzyme system of the cells plays a central role in the modification. For example, lipoxygenase enzymes produce lipid hydroperoxides, compounds that might be obligatory intermediates in LDL oxidative modification. Therefore, we asked the question: can a purified lipoxygenase directly modify LDL? In this report we show that purified soybean lipoxygenase, in the presence of phospholipase A_2, can cause biological modification of LDL. This modification is not dependent on the presence of cells, cell culture medium, or added transition metals.

Abbreviations: LDL, low density lipoprotein; PtdCho, phosphatidylcholine; TBARS, thiobarbituric acid-reactive substances; apoB, apoprotein B of LDL.

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MATERIALS AND METHODS

Materials

Cell culture media and supplies were from Gibco Laboratories, Grand Island, NY. Carrier-free $Na^{125}I$ was from Amersham. The soybean lipoxygenase (EC 1.13.11.12) was from Sigma, Type V, affinity-purified. The preparation was estimated to be greater than 90% pure by SDS polyacrylamide gel electrophoresis (data not shown). The phospholipase A_2 (EC 3.1.1.4) was purified from snake venom (*Naja naja naja*), and was a gift from Dr. E. Dennis, University of California, San Diego. Bee venom phospholipase A_2-conjugated agarose resin (4 U/mg of agarose) was from Sigma, St. Louis, MO.

Cells

A line of rabbit aortic endothelial cells was maintained in Ham's F-10 medium containing 15% fetal bovine serum, 10 ng/ml of epidermal growth factor, and 0.05 mg/ml gentamycin. Cells were used at confluence, and were washed three times in F-10 prior to addition of LDL. Normal human fetal foreskin fibroblasts were grown in DME medium containing 10% fetal bovine serum and 0.05 mg/ml gentamycin. When the cells were 60–80% confluent they were switched to DME containing 5 mg/ml of protein from human lipoprotein-deficient plasma and 0.05 mg/ml gentamycin. After 24 hr, lipoprotein degradation assays were done in fresh, identical medium containing 20 μg/ml of lipoprotein. Mouse resident peritoneal macrophages were isolated by peritoneal lavage, and 10^6 cells were put into each well of 24-well dishes in DME medium containing 10% fetal bovine serum, 50 μg/ml gentamycin, and 2.5 μg/ml fungizone. After overnight incubation, nonadherent cells were removed by washing the wells three times with DME medium, and then degradation assays were done immediately in DME medium.

Lipoprotein isolation and modification

LDL was isolated from the plasma of normal volunteers in the density range 1.019–1.063 g/ml by ultracentrifugation as previously described (17). EDTA (0.01%) was present throughout the isolation and dialyses to prevent oxidation. LDL was radioiodinated using Iodogen (18). LDL concentrations are given as mass of protein per ml. LDL was acetylated using acetic anhydride as described by Basu et al. (19). LDL (100 μg/ml) was oxidatively modified by confluent endothelial cells in Ham's F-10 medium for 24 hr, or by 0.020 mM copper acetate in F-10 medium for 24 hr at 37°C. The modified LDL was used directly in macrophage degradation assays. The specific activity of the ^{125}I-labeled LDL was approximately 40 cpm/ng.

Cellular degradation assays

Macrophages in 24-well plates were washed three times with DME medium, and then degradation assays were performed in a final volume of 0.5 ml of DME containing lipoprotein and no other additions. After 5 hr the medium was assayed for trichloroacetic acid-soluble, silver nitrate-soluble (non-iodide) radioactivity as described (14). Control incubations in wells containing no cells were performed and these control values were subtracted from the experimental values. The macrophage protein in the wells was determined after dissolving the cells in 0.2 N NaOH. Data are expressed as μg of apoB degraded during the 5 hr per mg of macrophage protein. Absolute values varied somewhat from experiment to experiment, but the relative degradation of native and various oxidized lipoproteins was reproducible. Fibroblasts were grown and switched to lipoprotein-deficient plasma as described above, then washed with DME and finally incubated with DME containing 20 μg/ml of ^{125}I-labeled lipoprotein and 5 mg/ml of protein from lipoprotein-deficient plasma. After 5 hr, the medium was removed and assayed for acid-soluble radioactivity as described above.

Electrophoresis and chemical assays

LDL was delipidated and the apoB was electrophoresed on SDS 3–14% gradient polyacrylamide gels as previously described (14). LDL holoparticles were examined by agarose gel electrophoresis at pH 8.6 as previously described (20). Protein was determined as described by Lowry et al. (21). TBARS were measured by mixing 50 μg of LDL with 6.7 mg of thiobarbituric acid and 150 μg of trichloroacetic acid in a final volume of 1.8 ml. This was boiled for 30 min, centrifuged, and the absorbance of the supernatant was measured at 532 nm. A standard curve was produced by using tetramethoxypropane to generate malondialdehyde in the assay. Data are expressed as nmol of malondialdehyde equivalents per mg of LDL protein.

RESULTS

Effect of lipoxygenase plus phospholipase A_2 on LDL

To test the possibility that lipoxygenase activity might biologically modify LDL, we first incubated ^{125}I-labeled LDL with purified soybean lipoxygenase alone in borate buffer, pH 9.0, for 24 hr at 37°C, and then assayed for macrophage degradation of the particle. There was no increase in degradation of the treated LDL (**Table 1**). The lipids from the lipoxygenase-treated LDL were extracted and saponified, and the fatty acid fraction was analyzed by thin-layer chromatography. Little or no oxidized fatty acid was present, implying that soybean lipoxygenase fails to

TABLE 1. Incubation of LDL with lipoxygenase plus phospholipase A_2 causes peroxidation and biological modification

	TBARS	Macrophage Degradation
	nmol/mg of apoB	*μg of apoB degraded per 5 hr per mg of protein*
Incubations		
Lipoxygenase plus phospholipase	15.3	2.6
Lipoxygenase alone	4.0	0.37
Phospholipase alone	2.3	0.95
No enzymes	2.0	0.69
Enzymes mixed with LDL immediately prior to assay	3.9	0.49
Controls		
Native LDL	1.7	0.64
Copper-oxidized LDL	65.0	4.6

LDL (0.5 mg of protein/ml) was incubated in 50 mM borate, pH 9.0, containing 0.45% NaCl, 1 mM $CaCl_2$, and 0.1 mM EDTA for 24 hr at 37°C. In some cases, soybean lipoxygenase (20 μg/ml) and/or snake venom phospholipase A_2 (0.1 μg/ml) were present. To ensure availability of oxygen for the lipoxygenase reaction, a large surface area-to-volume ratio was maintained by performing the incubations in 0.6 ml in 16 × 100 mm tubes with no caps. Each sample was sterile-filtered prior to assay. Macrophage degradation assays were performed using 10 μg/ml of lipoprotein.

act on the fatty acids of PtdCho when presented in an LDL particle. However, the purified enzyme did catalyze the oxidation of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine in the presence but not in the absence of deoxycholate (data not shown). Deoxycholate alone did not cause the oxidation. This effect of deoxycholate on enzyme activity confirms that the physical state of phospholipid substrate is critically important for lipoxygenase action. Since the best substrate for lipoxygenase is free fatty acid, we incubated LDL in the presence of lipoxygenase plus purified phospholipase A_2, reasoning that the phospholipase A_2 would release free fatty acids from the LDL PtdCho, and that these fatty acids would then serve as substrate for lipoxygenase, and possibly initiate oxidation of the LDL. This approach was successful, as shown in Table 1. When LDL was incubated with both soybean lipoxygenase and phospholipase A_2, there was production of TBARS and a dramatic increase in the degradation of the LDL by mouse peritoneal macrophages. Neither enzyme alone was effective in causing this modification (Table 1). Furthermore, when the two enzymes were mixed with LDL immediately prior to the macrophage degradation assay, there was no effect on macrophage degradation (Table 1). In other words, the enzymes must have an opportunity to act on the LDL, i.e., the increased degradation was not due to some possible effect on macrophage cellular processes. LDL modified by purified lipoxygenase plus phospholipase A_2 will hereafter be referred to as "enzymatically modified LDL."

The modification of LDL by lipoxygenase plus phospholipase A_2 also caused an increase in mobility on agarose gel electrophoresis (**Fig. 1**). Lipoxygenase alone had no effect; phospholipase A_2 alone caused some increase, presumably because of the extra negative charge carried by the free fatty acid products of phospholipase A_2 action. This effect of free fatty acids on LDL mobility has been noted previously (22). The presence of free fatty acids in the phospholipase A_2-treated LDL was confirmed by thin-layer chromatography, as shown in **Fig. 2**, lane D. LDL treated with both enzymes showed a small amount of unoxidized free fatty acid, and a larger amount of oxidized fatty acid (Fig. 2, lane C).

Lipoxygenase-mediated modification of LDL causes apoB breakdown

Oxidative modification of LDL by endothelial cells or by copper ion is accompanied by breakdown of the apoB moiety (14). As shown in **Fig. 3**, this is also true for enzymatically modified LDL, although fewer lower molecular weight fragments (less than 100 kDa) are produced by the purified enzymes than by endothelial cell modification. This experiment was performed with ^{125}I-labeled LDL because apoB from oxidized LDL does not stain well with Coomassie blue (13). Neither enzyme alone was effective (Fig. 3), demonstrating that the apoB breakdown was not due to contaminating protease activity.

Enzyme action on LDL can be sequential

Enzymatic modification of LDL was performed in two steps as follows. LDL was first incubated with bee venom phospholipase A_2 covalently coupled to agarose, and then the resin was removed by centrifugation. The treated LDL, containing free fatty acids (data not shown), was then treated with lipoxygenase and this effected oxidative modification, as shown in **Table 2**. Control incubations did not cause oxidative modification. These results show that the action of the two enzymes on LDL can be sequential, provided the phospholipase A_2 is allowed to act first.

Fig. 1. Effect of enzyme incubation with LDL on agarose gel electrophoretic mobility. ^{125}I-labeled LDL was incubated in buffer only (lane A), with lipoxygenase alone (lane B), phospholipase A_2 alone (lane C), or lipoxygenase plus phospholipase A_2 (lane D), for 24 hr as described in Table 1. Agarose gel electrophoresis was then carried out as previously described (20), the gel was dried, and autoradiographed. Lane E, native LDL; lane F, copper-oxidized LDL.

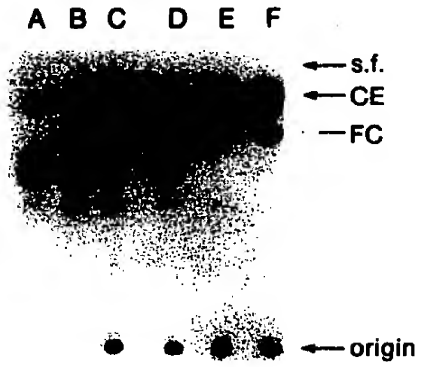

Fig. 2. Silica gel thin-layer chromatographic analysis of lipids from LDL treated with lipoxygenase and/or phospholipase A_2. LDL was incubated with lipoxygenase and/or phospholipase A_2 as described in Table 1. LDL (0.10 mg of protein) was then extracted by the method of Bligh and Dyer (23), with the pH adjusted to 4.0 with citric acid. The lower (chloroform) phase was removed, and the upper phase was re-extracted with fresh chloroform. The two chloroform samples were pooled, dried under nitrogen, redissolved in chloroform–methanol 1:1, and applied to a silica gel thin-layer plate. The plate was developed in the solvent system chloroform–methanol–water–acetic acid 180:20:1:1. Lipids were visualized by exposure to iodine vapor. To produce a standard for oxidized fatty acids, 200 nmol of linoleate was incubated with 10 μg of soybean lipoxygenase in 0.2 ml of 50 mM sodium borate, pH 9.0, for 1 hr at 37°C. This was then extracted as described for the LDL samples, and spotted in lane B. Linoleate with no lipoxygenase added was carried through the same procedure (lane A). Notice that native linoleate stains darker with iodine than does oxidized linoleate. The LDL samples had been treated with enzymes as follows: lane C, treatment with lipoxygenase and phospholipase A_2; lane D, phospholipase A_2 alone; lane E, lipoxygenase alone; lane F, no enzymes added. The iodine-staining material at the origin is probably phospholipid. Abbreviations: CE, cholesteryl ester; FC, free cholesterol; s.f., solvent front.

Macrophage recognition of enzymatically modified LDL

The macrophage scavenger receptor binds a variety of ligands, including acetylated LDL (5), endothelial cell-modified LDL (6), and maleylated albumin (3). Polyinosinic acid inhibits ligand binding to this receptor (3). To determine whether the scavenger receptor was involved in the macrophage degradation of enzymatically modified LDL, we performed competition experiments using enzymatically modified ^{125}I-labeled LDL, endothelial cell-modified ^{125}I-labeled LDL, and acetylated ^{125}I-labeled LDL as ligands, and unlabeled enzymatically modified LDL, endothelial cell-modified LDL, acetylated LDL, and polyinosinic acid as competitors. To avoid any possible interactions of phospholipase A_2 with lipoproteins during the macrophage incubations, we used immobilized phospholipase A_2 in the production of the enzymatically modified LDL (c.f. Table 2). The results of the competition experiments are presented in Figs. 4–6. **Fig. 4** and **Fig. 5** show that all four competitors inhibit the degradation of enzymatically modified LDL and endothelial cell-modified LDL. **Fig. 6** shows that the two oxidized LDLs inhibit the degradation of acetylated ^{125}I-labeled LDL; however, they do so poorly as compared to the homologous ligand, acetylated LDL. In summary, as judged by macrophage recognition, enzymatically modified LDL and endothelial cell-modified LDL are very similar ligands, but both of these are somewhat different from acetylated LDL. The difference may be due to differences in affinities or may reflect the existence of more than one class of scavenger receptor, a possibility supported by recent findings (25, 26).

Because the three ligands studied do not display mutual complete competition, it was important to show that the degradation of enzymatically modified LDL is mediated by a high-affinity, saturable process. **Fig. 7** shows the total degradation of each ligand as a function of lipoprotein concentration. All three ligands exhibit saturation for degradation. The results for endothelial cell-modified LDL and acetylated LDL agree with previously published work (3, 4).

Fibroblast recognition experiments

Enzymatically modified LDL was degraded by fibroblasts at less than 5% of the rate of native LDL (**Table 3**). Similar results were obtained with copper-oxidized LDL (Table 3). The same preparations showed increased degradation by macrophages (Table 3). The oxidative changes brought about by either method must result in loss of the

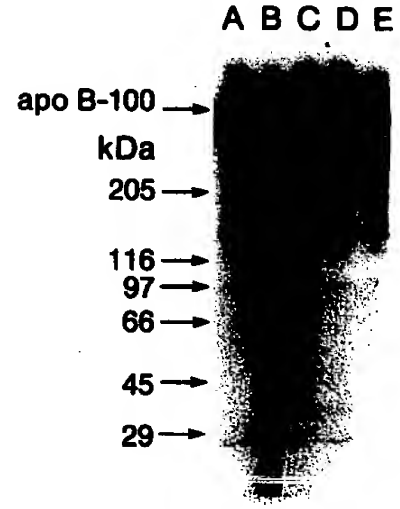

Fig. 3. Autoradiograph of SDS polyacrylamide gel electrophoretogram of apoB from ^{125}I-labeled LDL preparations treated with lipoxygenase and/or phospholipase A_2. ^{125}I-labeled LDL was incubated with lipoxygenase plus phospholipase A_2 (lane C), phospholipase A_2 alone (lane D), or lipoxygenase alone (lane E), for 24 hr as described in Table 1. The LDL was delipidated as previously described (14), and then 10 μg of apoB was loaded onto a 3–14% gradient polyacrylamide gel containing SDS. After electrophoresis, the gel was fixed, dried, and autoradiographed. Lane A, native LDL; lane B, endothelial cell-modified LDL. The bands below apoB-100 in native LDL may have arisen from partial kallikrein digestion during LDL isolation (24). The migrations of standards are indicated, and their M_r given in kDa. The standards used were, in order of decreasing mass: myosin, B-galactosidase, phosphorylase b, bovine serum albumin, egg albumin, and carbonic anhydrase.

TABLE 2. Enzyme action on LDL can be sequential

	TBARS	Macrophage Degradation
	nmol/mg of apoB	*μg of apoB degraded per 5 hr per mg of protein*
Incubations		
Phospholipase A_2–agarose, then lipoxygenase	10.4	6.1
Phospholipase A_2–agarose, then no other additions	3.2	1.8
"No resin" control, then lipoxygenase	2.4	0.8
"No resin" control, then no other additions	1.6	0.7
Controls		
Native LDL	2.6	1.2
Copper-oxidized LDL	28.0	7.8

LDL (0.5 mg/ml) in 0.7 ml of 50 mM sodium borate, pH 9.0, containing 0.45% NaCl, 1 mM $CaCl_2$, and 0.1 mM EDTA was incubated with 3 U of bee venom phospholipase A_2 covalently attached to beaded agarose (Sigma Chemical Co.) for 3 hr at 37°C. The mixture was rotated slowly in a sealed tube to ensure continuous suspension of the resin. A mock incubation with no resin added was a control. After 3 hr, the tubes were centrifuged to remove the resin, and to the supernatant was added purified lipoxygenase to 20 μg/ml. This was incubated at 37°C for 20 hr, and then the production of TBARS was determined; macrophage degradation assays were performed using 10 μg/ml of lipoprotein.

apoB/E receptor recognition site(s). This may occur via the oxidative loss of lysine residues (14), some of which are essential for apoB/E receptor binding (27).

DISCUSSION

These studies show that LDL can be oxidatively modified by the combined action of two purified enzymes, soybean lipoxygenase and phospholipase A_2. This modification occurs in the absence of cells or added transition metals, eliminating the many complex reactions that may take place in such systems, and focusing on peroxidation per se. This is the first demonstration of biological modification of LDL by a purified enzyme system, requiring no metals or cells. The results imply that the introduction of lipid hydroperoxides into an LDL particle can initiate biological modification of the lipoprotein. The creation of a pure enzymatic modification system may assist in the dissection of the essential chemical events of LDL oxidative modification.

Competition experiments indicate that macrophage degradation of enzymatically modified LDL proceeds along the same pathway as the degradation of endothelial cell-modified LDL (Figs. 4 and 5). Polyinosinic acid and acetylated LDL also inhibited the degradation of these two oxidized LDLs, implying that degradation was mediated by the scavenger receptor. However, in experiments testing the ability of lipoproteins to inhibit the degradation of acetylated ^{125}I-labeled LDL, neither enzymatically modified LDL nor endothelial cell-modified LDL were nearly as effective as acetylated LDL itself. The reason for this poor competition is not clear; however, it suggests a need for a re-evaluation of the idea that a single receptor mediates the degradation of all the modified LDL species recognized by

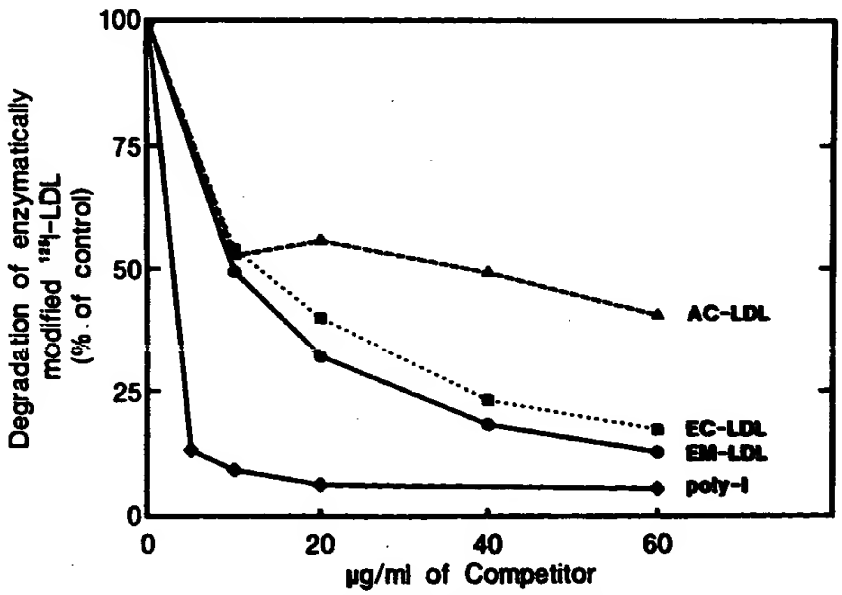

Fig. 4. Competition of macrophage degradation of enzymatically modified ^{125}I-labeled LDL by various unlabeled LDL preparations and polyinosinic acid. Macrophage degradation assays were performed using 5 μg/ml of enzymatically modified ^{125}I-labeled LDL as ligand and nonradioactive endothelial cell-modified LDL (EC-LDL; ■), acetylated LDL (AC-LDL; ▲), enzymatically modified LDL (EM-LDL; ●), or polyinosinic acid (poly-I; ◆) as competitor at the indicated concentrations. All points are the means of duplicate determinations.

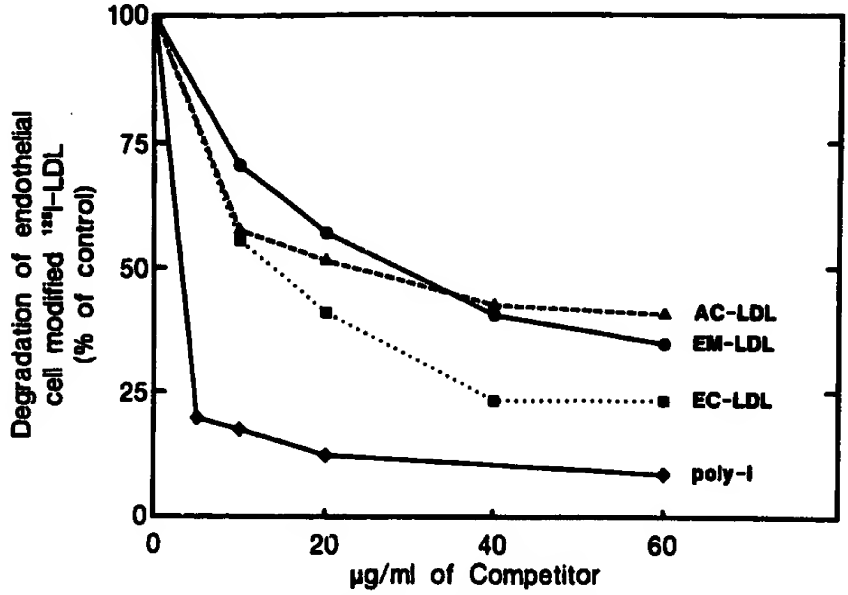

Fig. 5. Competition of macrophage degradation of endothelial cell-modified ^{125}I-labeled LDL by various unlabeled LDL preparations and polyinosinic acid. Macrophage degradation assays were performed using 5 µg/ml of endothelial cell-modified ^{125}I-labeled LDL as ligand and nonradioactive endothelial cell-modified LDL (EM-LDL; ■), acetylated LDL (AC-LDL; ▲), enzymatically modified LDL (EM-LDL; ●), or polyinosinic acid (poly-I; ♦) as competitor at the indicated concentrations. All points are the means of duplicate determinations.

the "scavenger receptor." We are currently investigating the possibility that there may be more than one class of scavenger receptor, as suggested in recent publications (25, 26).

LDL modification by lipoxygenase plus phospholipase A_2 differs from cell- or metal-mediated LDL oxidation in that the level of TBARS produced is much lower using the purified enzymatic system, although other features of the modification are similar. Recently, Hiramatsu et al. (10) reported that cultured human mononuclear cells can oxidize LDL, producing TBARS, but that the oxidized LDL so

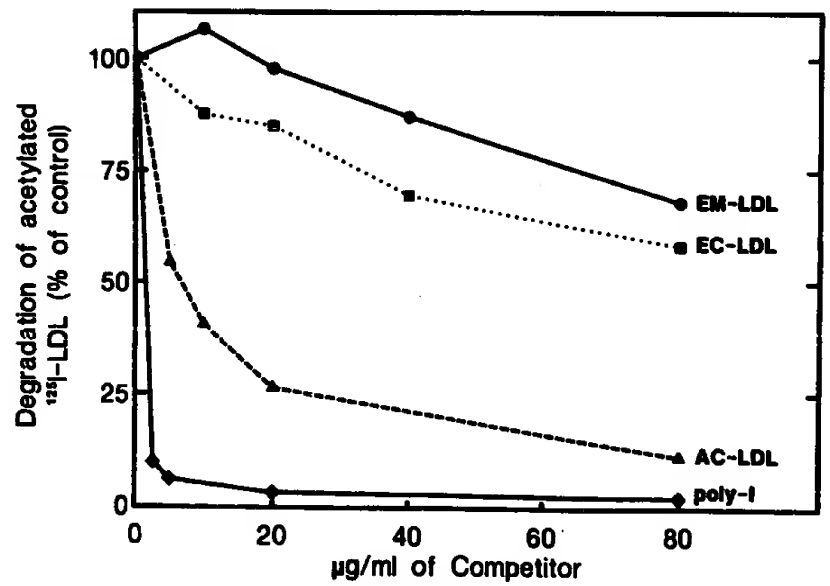

Fig. 6. Competition of macrophage degradation of acetylated ^{125}I-labeled LDL by various unlabeled LDL preparations and polyinosinic acid. Macrophage degradation assays were performed using 2 µg/ml acetylated ^{125}I-labeled LDL as ligand and nonradioactive endothelial cell-modified LDL (EC-LDL; ■), acetylated LDL (AC-LDL; ▲), enzymatically modified LDL (EM-LDL; ●), or polyinosinic acid (poly-I; ♦) as competitor at the indicated concentrations. All points are the means of duplicate determinations.

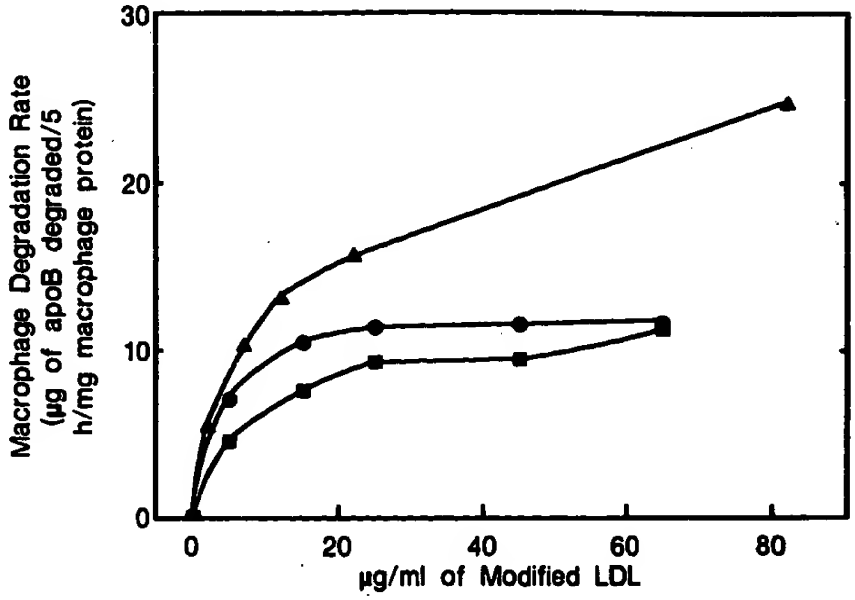

Fig. 7. Macrophage degradation of various LDL preparations as a function of concentration. Macrophage degradation assays were performed using acetylated ^{125}I-labeled LDL (AC-LDL; ▲), endothelial cell-modified ^{125}I-labeled LDL (EC-LDL; ■), or enzymatically modified ^{125}I-labeled LDL (EM-LDL; ●) as ligand at the indicated concentrations. The data are from the competition experiments presented in Figs. 4–6. All points are the means of duplicate determinations.

produced does not stimulate cholesteryl ester synthesis by macrophages, and thus is presumably not recognized by the scavenger receptor. Although peroxidation and biological modification of LDL are closely associated, taking place concurrently in a number of systems, the level of TBARS is not a reliable quantitative indicator of the degree of biological modification of LDL (i.e., macrophage degradation). It is possible that the production of TBARS during LDL oxidation is a side reaction on the overall pathway to production of the structure recognized by the scavenger receptor. It has been shown that the delipidated apoB of oxidized LDL contains the scavenger receptor binding structures (28). Perhaps an early event in LDL oxidation is the production of fatty acid hydroperoxides. Fatty acid hydroperoxides may directly cause oxidation of apoB, leading to generation of the structures recognized by the scavenger receptor. This reaction might be crucial to biological modification, and it might or might not lead to the production of TBARS. Moreover, the exact fate of the oxidized lipids may be very different in different modification systems, some conditions favoring production of TBARS and some inhibiting their production. In summary, evidence suggests that the level of TBARS, although it often parallels biological oxidation of LDL, is not a reliable quantitative indicator of modification.

How does lipoxygenase plus phospholipase A_2 cause modification of LDL? We have shown (Table 2) that the phospholipase A_2 must act first. Presumably its role is to produce a good substrate for the lipoxygenase, which then can initiate oxidation by introducing fatty acid hydroperoxides into the LDL particle. These compounds are unstable, and may then decompose in a manner that causes oxidation of apoB and generation of the epitope recognized by the scavenger receptor. This may occur by direct reaction of the lipid hydroperoxides with apoB or via the generation of singlet oxygen. Kanofsky and Axelrod (29) have recently shown that singlet oxygen is produced during the reaction of soybean lipoxygenase with linoleate. Co-oxidation of apoB by lipoxygenase action on LDL lipids may have a precedent in the observation that lipoxygenase action on fatty acids can cause co-oxidation of carotene and other organic molecules (30). The role of the phospholipase A_2

TABLE 3. Enzymatically modified LDL is poorly degraded by fibroblasts

	Cellular Degradation	
	Macrophages	Fibroblasts
	µg of apoB degraded/5 hr per mg of protein	
Native LDL	1.0	2.2
Copper-oxidized LDL	6.5	0.09
Enzymatically modified LDL	2.4	0.10

LDL was incubated with lipoxygenase and phospholipase as described in Table 1, and then was used as a substrate for cellular degradation assays with macrophages and fibroblasts. Lipoproteins were present at 10 µg/ml for macrophages and 20 µg/ml for fibroblasts.

in the enzymatic modification of LDL is in contrast to the role of the LDL-associated phospholipase A_2 activity which has been shown to be necessary for LDL oxidation by cells (11). This LDL-associated activity requires oxidized phospholipid as substrate, and thus it acts only after LDL oxidation has begun.

Is endothelial cell lipoxygenase important in the cell-mediated oxidation of LDL? If so, is concurrent action of a phospholipase A_2 required? Endothelial cells do contain both enzyme activities (31, 32). Perhaps the endothelial cell lipoxygenase does not require free fatty acid as substrate, and therefore can act independently of a phospholipase. Indeed, it has been reported that the lipoxygenases from neutrophils (33) and reticulocytes (34) can act directly on PtdCho. Another possibility is that the role of the lipoxygenase may be to produce reactive compounds that are the key activators of LDL oxidation.

Endothelial cell-induced modification is only effective when Ham's F-10 medium is used. This may reflect primarily the presence of transition metal ions but could also reflect the presence of higher concentrations of sulfhydryl compounds, which Parthasarathy (12) has shown to accelerate lipid peroxidation strikingly. If cellular lipoxygenases play a role as discussed above, why is the nature of the medium important? We suggest that endothelial cell-induced modification is a two-step process. The first step would be lipoxygenase-mediated and result in the introduction of lipid peroxides into LDL. The second step might then be dependent upon the presence of metal ions or sulfhydryl compounds to facilitate propagation. Such a two-step model might help explain the variations in degree of modification seen with LDL preparations from different individuals (35). LDL preparations containing more lipid peroxides as isolated would be further oxidized more rapidly and/or more completely. In the artificial system utilized in the present studies, no second step is absolutely required. Presumably the use of pure enzymes at high concentrations permits introduction of large amounts of lipid peroxides into LDL—amounts sufficient in themselves to drive the overall oxidative modification. This two-step model can then account for the role of lipoxygenase and also explain the need for a medium containing transition metals and/or sulfhydryl compounds (11, 12).

Other investigators have suggested that superoxide plays an important role in the cell-induced oxidation of LDL (8, 10). The role of superoxide anion could relate to the described effect of peroxides to stimulate lipoxygenase activity (36). Destruction of superoxide by superoxide dismutase would then effectively decrease further lipoxygenase activity and block the initiation step postulated here. However, even if cellular lipoxygenases do play a role in cell-induced LDL modification, this does not exclude the possibility that other mechanisms are simultaneously operative. Further work will be needed to assess the possible relevance of lipoxygenases under in vivo conditions.

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